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ORIGINAL PAPER

Assessing Serra da Estrela PDO cheeses' origin-production date using fatty acids profiles

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Abstract

Serra da Estrela is a Portuguese traditional cheese produced with raw ewe's milk from "Churra Mondegueira" and "Bordaleira" autochthonous breeds and the wild thistle flower (*Cynara cardunculus* L.), which benefits from the status of Protected Designation of Origin. Cheese chemical composition, namely the fatty acids profile, depends on milk composition and on manufacturing practices. Thus, the identification of possible chemical biomarkers capable of classifying Serra da Estrela cheeses according to the dairy manufacturing plant, geographical origin or production date would be of utmost relevance for producers and consumers. A typical fatty acids profile, including 23 saturated and unsaturated fatty acids, was identified for the studied cheeses, being butyric, caproic, caprilic, capric, lauric, miristic, palmitic, stearic, oleic, linoleic and its trans-isomer and α-linolenic acids the most abundant ones (relative mean abundances ranging from 1.4% ± 0.5% to 23.9% ± 1.9%). Linear discriminant models were established based on the most discriminative fatty acids (namely, caproic, caprilic, undecanoic, lauric, pentadecanoic, palmitic, palmitoleic, heptadecanoic, oleic, linoleic trans-isomer, heneicosanoic and arachidonic acids) that included less abundant fatty acids, which were selected using the simulated annealing algorithm. The established models enabled assessing cheeses' origin (models based on 10–12 fatty acids) and/or production date (model based on 20 fatty acids) with predictive sensitivities of 71–88%. Therefore, fatty acids profiles coupled with chemometric techniques, could be foreseen as a fingerprint of cheese's genuineness, enhancing the consumers' confidence when purchasing this high-value cheese.

Keywords Serra da Estrela cheese · Fatty acids profile · Origin assessment · Linear discriminant analysis · Simulated annealing algorithm

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Introduction

Cheese is a source of essential nutrients and health-promoting compounds in the human diet, containing several fatty acids. Its nutritional value depends on the milk composition as well as on the cheese making conditions. Consumers perceive cheese as a food product rich in nutritionally controversial saturated fatty acids (SFA), which are associated to high cholesterol and cardiovascular diseases although recent studies show different tendencies. However, cheese is also a source of some health-promoting mono- and polyunsaturated fatty acids (MUFA and PUFA, respectively) as well as short chain fatty acids (SCFA) and certain trans-fatty acids that are considered as part of a healthy diet [1, 2].

Serra da Estrela is an ewe`s traditional Portuguese cheese that, since 1985, has a status of Protected Designation of Origin (PDO) with EU recognition [3], and so its production is regulated and geographically limited. Serra da Estrela cheese is produced from raw ewe's milk from "Churra Mondegueira" and "Bordaleira" Portuguese autochthonous breeds and coagulated using wild thistle flower (*Cynara cardunculus* L.), during a limited time period (usually from December to May). The cheese characteristics are legally defined, although many extrinsic factors (e.g., climate, nutritional and physiological status, lactation stage) may condition the milk chemical and microbiological characteristics and consequently the final cheese composition [4]. Serra da Estrela is the most known and popular Portuguese cheese and is appreciated worldwide, being preferentially consumed as a soft cheese, with an average maturation of 30–45 days, although some consumers prefer to consume it as a hard cheese after at least 6 months of storage [5–7].

Due to its social and agro-economic relevance, Serra da Estrela cheese has been the focus of several studies, including the improvement of the efficiency of the certified traditional cheese production system [8] or the evaluation of health and hygiene standards in local artisanal production of this cheese [9]. Correia et al. [10] showed that cardoon ecotype influences color, texture and sensorial characteristics of Serra da Estrela cheeses. Indeed, cardoon flower has an impact on cheesemaking yield and milk coagulation properties, as well as on milk clotting and proteolytic activities [11]. Other works addressed the (bio)chemical characterization of this PDO cheese, namely the assessment of the fatty acids profile and how it is influenced by different factors, such as ripening time, cheese making season, storage time-period, different cheese functionalizing treatments or even due to the sheep feed supplementation [4–7, 12–16]. The reference analytical technique used to quantify the fatty acids profile in milk and dairy products is based on gas chromatography (GC) analysis.

In the dairy industry, the use of low-priced milk in cheese production is a common fraudulent practice, and so, determining the milk origin is a crucial task, which may be accomplished using DNA based methods [17]. Also, the production of some traditional cheeses requires the use of raw ewe's milk, and so the possibility of detecting the use of thermized milk is of utmost relevance, which may be achieved by chromatographic techniques [18]. Similarly, Serra da Estrela cheese, is a dairy traditional product highly prone to different forms of adulteration, namely, the use of milk of non-allowed sheep breeds for the fraudulent production of this artisanal cheese. To detect this fraudulent practice, a randomly amplified polymorphic DNA method was recently developed [19]. However, to the authors' best knowledge, the possibility of using the fatty acids profile coupled with chemometric tools for discriminating among Serra da Estrela cheese producers, geographical origin within the limited PDO region or the cheese production date was never evaluated. On the other hand, the fatty acids content of Serra da Estrela cheeses depends on the dairy manufacturing plant, which could be due to the milk composition variability as well as to slight differences in the manufacturing protocols [12–15, 20]. In fact, milk fatty acids composition is highly dependent on animal feeding [21–23] and lactation stage [24], being possible, for example, to use the fatty acids composition to distinguish the milk obtained from animals subjected to feeding silage-free versus conventional feeding silage [21]. Fatty acids profiles have been successfully used as authentication markers in other food matrices such as fruit juices [25] and sweet peppers [26]. Thus, the present work aims to verify if the fatty acids profile would depend on those factors in such an extent that could enable its usage as chemical biomarkers for verifying the provenance of traditional Serra da Estrela cheeses. For that, a multivariate classification approach, based on linear discriminant analysis (LDA) coupled with the simulated annealing (SA) variable selection algorithm, was applied [27]. Indeed, this possibility would enable the cheese producers to have a straightforward chemical approach for ensuring cheese brand and age, and also providing the consumer a guarantee regarding the high-value product they are purchasing.

Materials and methods

Serra da Estrela cheese samples

Twenty-four Serra da Estrela cheeses (~ 1 kg), produced between November 2017 and March 2018, were collected after 45 days of maturation (from January to May 2018), at selected certified producers, and immediately transported, in refrigerated boxes, to the laboratory, being then split in different portions, which were frozen (− 40 °C) until analysis.

The cheeses were produced with milk, collected from ewes ("Churra Mondegueira" and "Bordaleira" autochthonous breeds) and acquired in six certified cheese producers (coded as Producer 1 to 6) located in five municipalities within the delimited PDO region (*Celorico da Beira*—CB, *Gouveia*—G, *Nelas*—N, *Oliveira do Hospital*—OH, and *Penalva do Castelo*—PC), belonging to three Portuguese districts, namely, Coimbra (OH, Producer 1), Guarda (CB, Producer 2; and G, Producer 5) and Viseu (N, Producer 6; and PC, Producers 3 and 4) districts (Portugal) as shown in Fig. 1. In total, 48 were independent samples studied (two samples per cheese), according to: Producer 1–5 cheeses × 2 collected in November 2017, December 2017, January 2018, February 2018 and March 2018; Producer 2–3 cheeses × 2 collected in November 2017, February 2018 and March 2018; Producer 3–3 cheeses × 2 collected in December 2017 and March 2018; Producer 4–4 cheeses × 2 collected in December 2017, February 2018 and March 2018; Producer 5–5 cheeses × 2 collected in November 2017, January 2018, February 2018 and March 2018; Producer 6–4 cheeses × 2 collected in November 2017, December 2017, February 2018 and March 2018. It should be stated that (*data not shown*), the studied cheeses had moisture contents varying from 44 to 52%, total fat levels ranging from 20 to 30%, total protein contents of 19 to 25% and salt contents between 1 and 2%.

Reagents

All reagents and solvents used were of analytical grade purity and acquired from different suppliers (Lab-Scan, Fluka, Merck, Panreac, Riedel-de-Haën, and Sigma-Aldrich). The fatty acids methyl ester reference standard mixture (C4–C24, FAME Mix Supelco 37) was from Supelco and purchased to Sigma-Aldrich. Deionized ultra-pure water was obtained from a Milli-Q50 equipment.

Lipids extraction

Lipids were extracted (two independent extractions for each cheese studied) following the International Standard Method described in ISO 14156/IDF 172:2001 [28], with some modifications. Briefly, 0.5 g of each cheese sample (48 samples = 24 cheeses × 2 independent samples) were weighed and placed into a 10 mL vial, to which 5 mL of *n*-hexane were added plus 1 mL of a KOH–methanol solution (5 mol/L). The mixture was then placed in an ultrasound bath during 5 min, after which it was allowed to stand for more 5 min at ambient temperature. This procedure was repeated three times, in order to ensure the formation of two immiscible phases. Then eight drops of glacial acetic acid were added and the mixture was manually shaken during 1 min. Then, the *n*-hexane phase was removed from the vial and filtered through a nylon filter (0.2 µm from Millipore), and frozen until being analyzed by gas-chromatography (GC).

Gas-chromatography analysis

GC analysis followed the method described by Dias et al. [29] with some adaptations. The fatty acid methyl esters (FAME) of each cheese extract (for each cheese studied, two extractions were performed being each injected twice) were analyzed using a GC 1000 instrument from DANI equipped with a split/splitless injector, a flame ionization detector

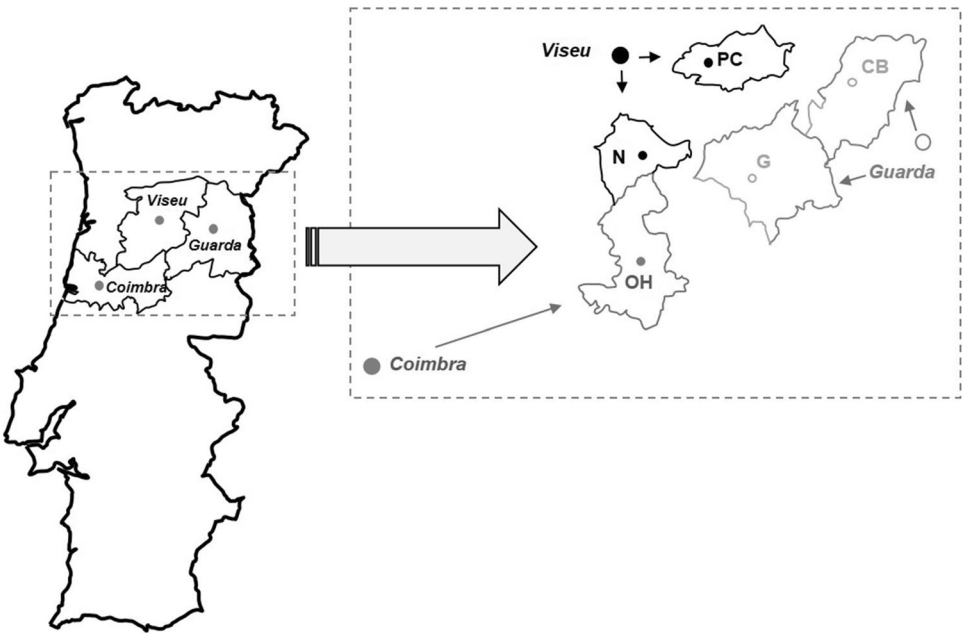

Fig. 1 Geographical origin of the Serra da Estrela PDO cheese samples evaluated (CB—*Celorico da Beira*, Producer 2; G—*Gouveia*, Producer 5; N—*Nelas*, Producer 6; OH—*Oliveira do Hospital*, Producer 1; and, PC—*Penalva do Castelo*, Producers 3 and 4)

(FID) and a Zebron, column (ZB-FAME from Phenomenex: (30 + 5) m × 0.25 mm ID × 0.20 µm). The oven temperature program was as follows: the initial temperature of the column was 100 °C, held for 2 min, then a 10 °C/min ramp was used until a 140 °C, followed by a 3 °C/min ramp until 190°, then a 30 °C/min ramp until reaching 260 °C and held for 2 min. The carrier gas (hydrogen) flow rate was 4.0 mL/min (0.61 bar), measured at 50 °C. Split injection (1:59) was carried out at 250 °C, being the detector at 260 °C. A constant flow rate of 1 mL/min was used. For each analysis 1 µL of the sample was injected in GC equipment. The identification was carried out by comparing the relative retention times of the FAME to the commercial standard [reference sample of FAME Mix Supelco 37 (C4–C24)]. The quantification was achieved through CSW 1.7 (DataApex 1.7, Prague, Czech Republic). The results were expressed in relative percentage of each fatty acid.

Statistical analysis

Linear discriminant analysis (LDA) was used to evaluate the possibility of using the Serra da Estrela fatty acids profiles to discriminate between the six cheese producers (Producers 1 to 6), the five geographical origins (CB, G, N, OH and PC municipalities) and the five production dates (November 2017 to March 2018), using the PDO cheeses, which experimental details were previously described in subsection "Serra da Estrela cheese samples". Furthermore, to determine the best sub-set of variables (within the individual fatty acids detected plus the SFA, MUFA and PUFA contents) the Simulated Annealing (SA) meta-heuristic variable selection algorithm was applied [30–32]. As previously described [27], the SA algorithm selects the optimal conditions based on the annealing physic process. The algorithm allows identifying a subset of variables among the original independent variables, which would enable to get a global optimum for a given approximation criterion, selected within a large search space of other possible subsets of variables. In detail, the algorithm searches a global minimum for optimizing a system with *k* variables. In each iteration, the values the current subset of variables *k* and the new subset to be tested (also with k variables) are compared by applying a test that measures the quality of the model performance achieved with those two subsets of variables. The new solution is randomly selected in the neighborhood of the current solution and tested according to the rules of the SA, being selected the current solution if it gives better results than the initial one. The algorithm continues the search for new solutions till it reaches the maximum number of attempts established at the beginning of the procedure. In general, 10,000 attempts are used to select the best subset of variables (best model), starting the process of selecting the best subsets of variables on each trial, thus ensuring a greater confidence in finding a true optimal solution [31, 33]. In this work, for each subset of variables under evaluation [combinations of 2 to 25 variables within the 26 possible variables (23 fatty acids plus SFA, MUFA and PUFA)], the sub-set chosen was the one that enabled to obtain the maximum percentage of correct classifications with the minimum number of variables, for the leave-one-out cross-validation (LOO-CV) procedure. This internal validation variant, although being considered an over-optimistic technique, has been widely used when the number of samples within each group that is being studied, is limited and so, it is not possible to use an independent external validation group. To normalize the weight of each variable in the final linear classification model, variable scaling and centering procedures were implemented. The classification performance of each LDA model was graphically evaluated using 3-D plots of the three most significant linear discriminant (LD) functions as well as by calculating the sensitivity values (i.e., the percentage of correctly classified samples according to the pre-established data groups). All statistical analysis was performed using the Subselect [31, 33] and MASS [34] packages of the open source statistical R program (version 2.15.1), at a 5% significance level.

Results and discussion

Serra da Estrela fatty acids profile

The fatty acids profile of 24 × 2 Serra da Estrela cheese samples are shown in Table 1. As can be inferred (Fig. 2), 23 individual fatty acids could be detected and quantified in all cheese samples, including 15 saturated fatty acids (SFA: $C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{11:0}$, $C_{12:0}$, $C_{13:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:0}$, $C_{20:0}$, $C_{21:0}$ and $C_{22:0}$), four monounsaturated fatty acids (MUFA: $C_{14:1}$, $C_{16:1}$, $C_{18:1n9c}$ and $C_{20:1}$) and four polyunsaturated fatty acids (PUFA: $C_{18:2n6t}$, $C_{18:2n6c}$, $C_{18:3n3}$ and $C_{20:4n6}$). The most abundant fatty acids detected in the cheese samples analyzed were $C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1n9c}$, $C_{18:2n6t}$, $C_{18:2n6c}$ and $C_{18:3n3}$, which relative abundances are in agreement with those previously reported by Carocho et al. [5, 12, 13] for this type of Protected Designation of Origin (PDO) cheese, although the cheeses were produced by a different manufacturing plant located in a different geographical region (Seia municipality, Portugal). It should be remarked that, SFA represented ~ 74% of the relative fatty acid abundance, followed by MUFA, which content was ~ 20% and by PUFA with a content of ~ 6%. Finally, it should be referred that the overall fatty acids profile established in the present study was in line with other previous reports for Serra da Estrela cheeses [6, 7, 12, 13, 16]. Moreover, compared to other cheeses produced from ewe milk, similar fatty acids profiles were obtained although the individual relative abundances could

Table 1 Fatty acids profile (relative abundance, %), determined by GC-FID, of *Serra da Estrela* cheeses (mean ± standard deviation) produced at five geographical origins (CB—Celorico da Beira, G—Gouveia, N—Nelas, OH—*Oliveira do Hospital* and PC—*Penalva do Castelo* municipalities) located within the delimited PDO region, by six different local certified producers (Producer 1 to 6) at five production dates (November 2017 to March 2018)

Fatty acids	Serra da Estrela cheeses ($n = 24$ cheeses × 2 independent samples)	
	Mean content ± standard deviation (%)	Minimum–maximum range (%)
$C_{4:0}$ (butyric acid)	4.3 ± 1.4	0.5–6.4
$C_{6:0}$ (caproic acid)	3.6 ± 1.0	1.1–6.2
$C_{8:0}$ (caprilic acid)	3.2 ± 0.9	1.7–6.1
$C_{10:0}$ (capric acid)	8.6 ± 2.2	4.1–14.9
$C_{11:0}$ (undecanoic acid)	0.10 ± 0.04	0.03–0.24
$C_{12:0}$ (lauric acid)	5.2 ± 1.1	3.3–7.9
$C_{13:0}$ (tridecanoic acid)	0.09 ± 0.02	0.04–0.14
$C_{14:0}$ (miristic acid)	11.4 ± 1.3	7.6–13.7
$C_{14:1}$ (miristoleic acid)	0.30 ± 0.14	0.13–0.56
$C_{15:0}$ (pentadecanoic acid)	1.0 ± 0.2	0.5–1.4
$C_{16:0}$ (palmitic acid)	23.9 ± 1.9	18.7–26.8
$C_{16:1}$ (palmitoleic acid)	0.8 ± 0.2	0.2–1.0
$C_{17:0}$ (heptadecanoic acid)	0.7 ± 0.1	0.4–1.0
$C_{18:0}$ (stearic acid)	10.8 ± 1.8	6.9–13.9
$C_{18:1n9c}$ (oleic acid)	19.1 ± 3.8	11.2–27.0
$C_{18:2n6t}$ (linoleic trans-isomer acid)	1.4 ± 0.5	0.4–2.3
$C_{18:2n6c}$ (linoleic acid)	3.1 ± 0.5	2.4–4.1
$C_{18:3n3}$ (α-linolenic acid)	1.5 ± 0.4	0.4–2.3
$C_{20:0}$ (arachic acid)	0.23 ± 0.06	0.12–0.40
$C_{20:1}$(eucosenoic acid)	0.09 ± 0.05	0.02–0.30
$C_{21:0}$ (heneicosanoic acid)	0.07 ± 0.05	0.02–0.28
$C_{20:4n6}$ (arachidonic acid)	0.3 ± 0.1	0.01–0.5
$C_{22:0}$ (behenic acid)	0.11 ± 0.06	0.01–0.2
ΣSFA	73.4 ± 4.2	64.8–81.9
ΣMUFA	20.3 ± 3.8	12.4–28.1
ΣPUFA	6.3 ± 0.7	4.3–7.5

SFA saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids

be different but within the same order of magnitude, like semi-hard uncooked Italian cheese [35], Manchego Spanish cheese [36], Kashkaval Greek cheese [37], Tulum Turkish cheese [38], Chilean, French and Spanish commercial cheeses [39], Serbian white brined cheeses [1] or of hard ewe milk cheeses [40].

Nevertheless, from the data given in Table 1, it is evident the variability of the relative abundances of each detected fatty acid (high minimum–maximum relative ranges). This variation may be attributed to the fact that the studied cheeses were obtained from different producers, placed within the PDO region but at different geographical locations (municipalities) and produced at different time-periods (between November 2017 and March 2018). Indeed, it is known that, different climatic conditions, forage species, pasture and phenological phase influence ewe's milk fatty acids profile and thus, of the related cheeses, which is of main relevance since fatty acid composition is responsible by the cheese flavor nuances [35].

Fatty acids profile as a biomarker fingerprint for assessing Serra da Estrela cheese origin (producer or geographical location) and production date

The possibility of using the fatty acids profiles as a biomarker fingerprint for classifying Serra da Estrela cheeses according to the producer/geographical origin or the production date was further investigated. For that, the fatty acids profiles per producer, per geographical origin or per production date were established (*data not shown*) and used instead of the 'average' fatty acid profile shown in Table 1. To the authors' best knowledge, no previous work addressed this topic for Serra da Estrela PDO cheeses. Nevertheless, in the literature it was demonstrated that fatty acids together with mineral composition (macro and micro elements) allowed discriminating Serra da Estrela cheeses according to the type of plant species used as natural preserving agents as well as the cheese functionalizing method, using a linear discriminant analysis coupled with a stepwise variable selection method (LDA-stepwise model) [12, 13]. Regarding other types of ewe cheeses, the fatty acids profiles were successfully used to group "Terrincho" cheeses (a Portuguese PDO cheese) according to the dairy production plant by applying Principal Component Analysis (PCA) [41], to discriminate (LDA-stepwise model) cheeses according to different sheep daily dietary treatments (corn grain or beet pulp based supplementation) [35] or, for example, to classify (PCA) commercial ewe cheeses according to the geographical origin (country of origin) [39]. Also, it was shown that the unsaturated fatty acids were suitable markers to differentiate pure cow's, ewe's and goat's milk cheeses and cheeses obtained using mixtures of the referred milks, being using a canonical biplot statistical approach [42].

So, first the fatty acids profiles were used aiming to verify their capability to discriminate Serra da Estrela cheeses according to the cheese producer (Producer 1 to 6), regardless the geographical origin or the cheese production date. Indeed, it has been reported that the cheese fatty acids composition and their relative abundance can be influenced by the different manufacturing protocols of each cheese producing plant [14, 20]. In this study, a linear discriminant analysis-simulated annealing (LDA-SA) model, with five significant discriminant functions

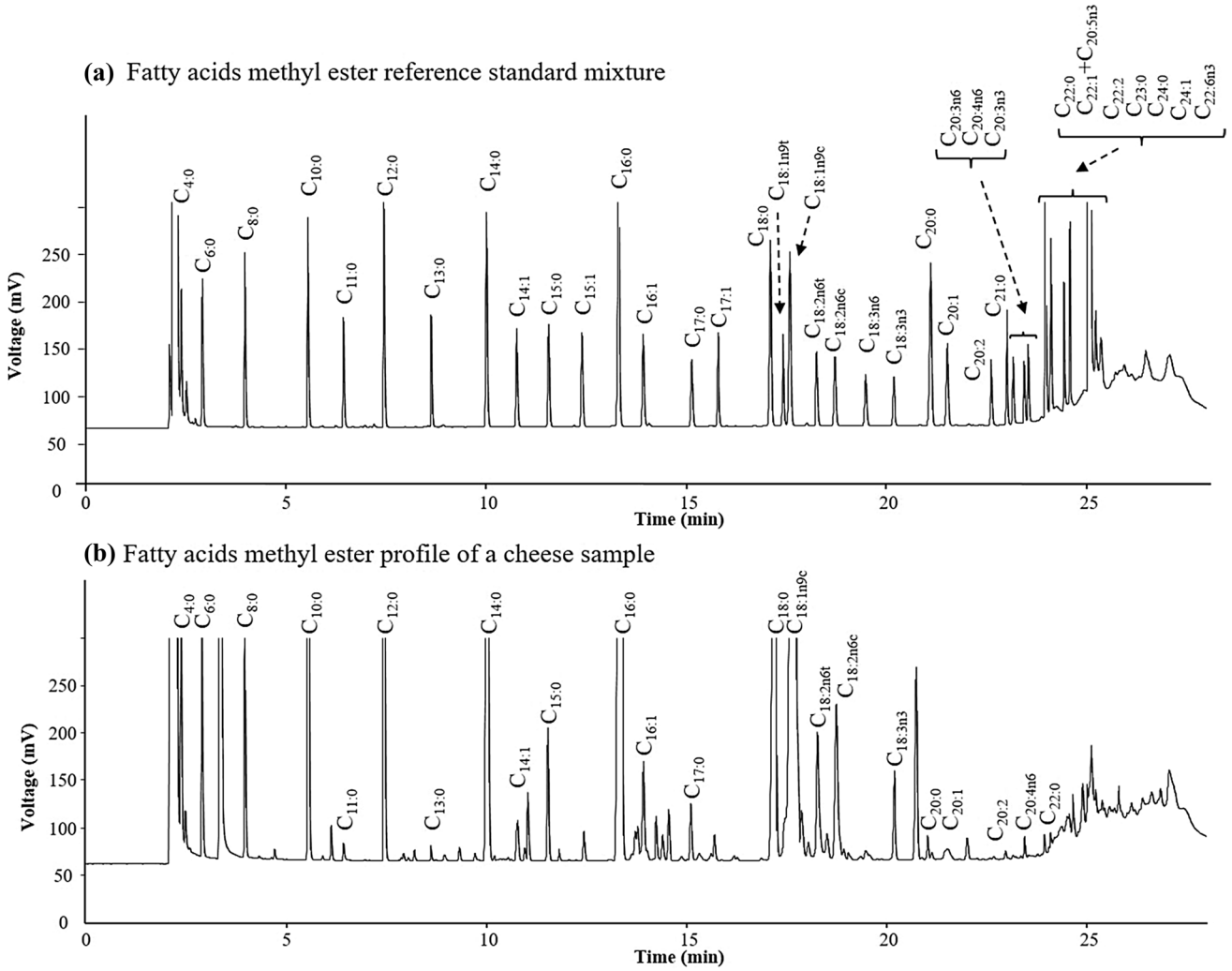

Fig. 2 Typical GC fatty acids methyl ester chromatograms: **a** reference standard mixture (C4–C24), **b** Serra da Estrela cheese (cheese sample from Producer 4, located at *Penalva do Castelo* and produced in February of 2018)

(explaining the first three functions 97% of the total variance) was established based on the experimental relative abundance of ten fatty acids ($C_{10:0}$, $C_{11:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{16:1}$, $C_{17:0}$, $C_{18:2n6t}$, $C_{20:1}$ and $C_{21:0}$). It should be noticed that the variables selected by the SA algorithm as the most discriminative ones (among the 23 identified fatty acids plus the total SFA, MUFA and PUFA, i.e., ΣSFA, ΣMUFA and ΣPUFA, respectively) included six of the less abundant fatty acids. The classification multivariate linear model allowed correctly classifying 92% of the original grouped data (Fig. 3) as well as 81% of the samples using the LOO-CV procedure. For the latter internal-validation technique the individual selectivity (true positive percentage or probability of detection) varied from 50 to 100% and the individual specificity (true negative percentage) ranged from 70 to 100%. The overall results were satisfactory, showing the practical possibility of using the fatty acids profiles as screening tool to discriminate cheese producers.

Since two of the producers were from the same municipality (geographical origin), the potential use of the fatty acids profile to identify the geographical origin (five municipalities within the delimited PDO region) of the Serra da Estrela PDO cheeses was also evaluated. A LDA-SA model, with four significant discriminant functions (which three first ones explained 98% of the data variability), was obtained based on the relative abundance of 12 fatty acids ($C_{6:0}$, $C_{8:0}$, $C_{11:0}$, $C_{12:0}$, $C_{15:0}$, $C_{16:0}$, $C_{16:1}$, $C_{17:0}$, $C_{18:1n9c}$, $C_{18:2n6t}$, $C_{21:0}$ and $C_{20:4n6}$) and the ΣMUFA. Once again, the model included some of the less abundant fatty acids, as best discriminant variables. It should also be remarked that, compared to the previous LDA-SA model, seven fatty acids are common to both classification models. Furthermore, the total MUFA content appears as one of the most

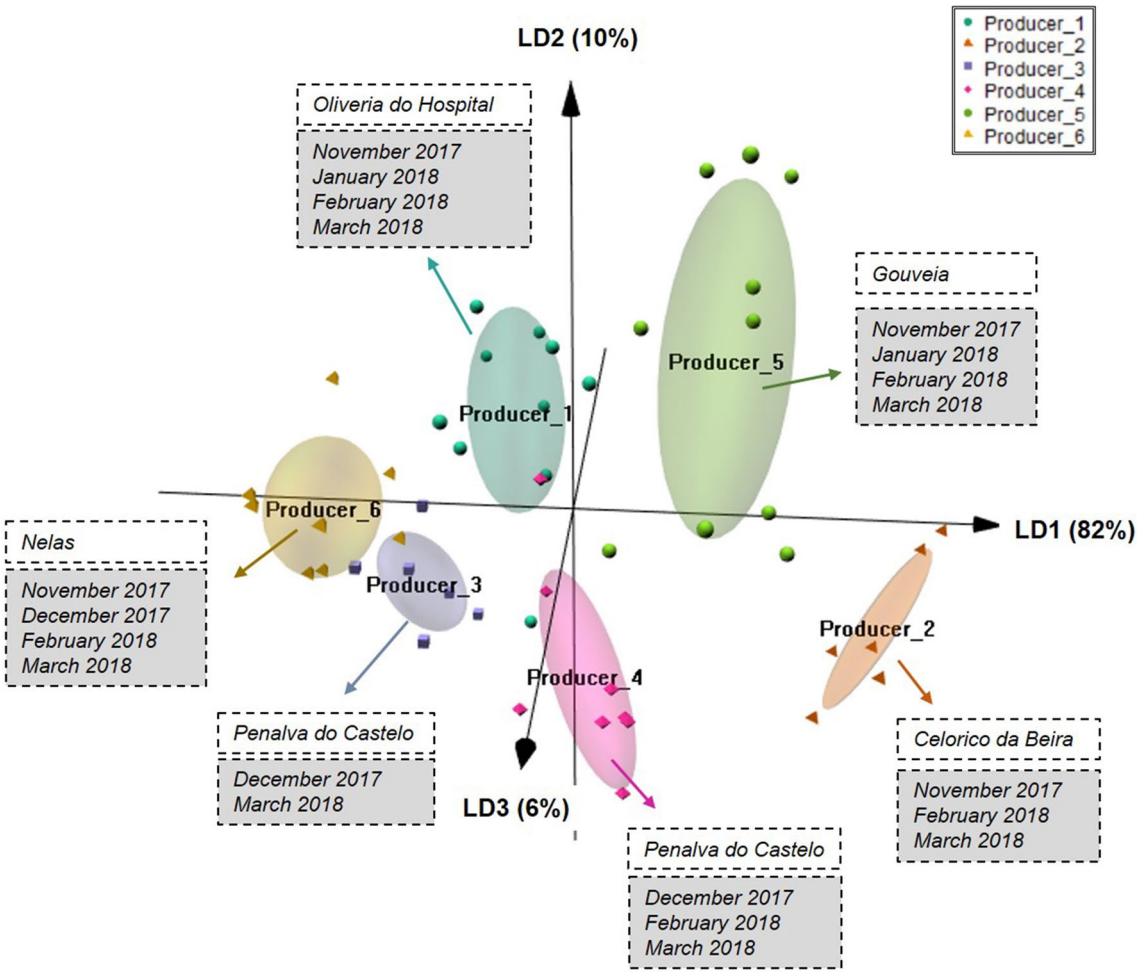

Fig. 3 LDA-SA model classification of Serra da Estrela PDO cheeses (24 cheeses × 2 independent samples) according to the cheese producer (Producer 1 to 6), independently of the production geographical origin (five municipalities within the delimited PDO region) or the production date (five months, ranging from November 2017 to March 2018): 3D plots of the first three most significant discriminant functions based on relative abundance of ten fatty acids ($C_{10:0}$, $C_{11:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{16:1}$, $C_{17:0}$, $C_{18:2n6t}$, $C_{20:1}$ and $C_{21:0}$) selected using the SA algorithm

discriminant variables, showing that the geographical origin (and indirectly the pasture and sheep diets) plays a key role on the MUFA cheese levels. The LDA-SA model established allowed a global correct classification of 96% of the original grouped samples (Fig. 4) and of 88% for the leave-one-out cross-validation (LOO-CV) procedure (individual true positive percentages of 80–100%; and, true negative percentages varying between 82 and 100% for LOO-CV). The satisfactory true positive and negative rates obtained (overall and individual per group), may allow stating that the geographical origin would have a major relevance than that of the cheese producer itself, and that the fatty acids profile may be foreseen as a geographical origin biomarker fingerprint.

The discrimination performance of the fatty acids profiles for classifying the Serra da Estrela cheese samples taking into account the cheese production dates (November 2017 to March 2018, independently of the cheese producer or the geographical origin) was further investigated. Seasonal changes of the fatty acids profile are expected and were reported, for example, by Hirigoyen et al. [2] for Uruguayan "Colonia" cheeses, produced with cow milk during autumn and spring. Using the proposed chemometric approach, a LDA-SA model with four significant discriminant functions (explaining the first three functions 98% of the data variability) was developed. The model was based on 20 fatty acids ($C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{11:0}$, $C_{12:0}$, $C_{13:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:1}$, $C_{17:0}$, $C_{18:0}$, $C_{18:1n9c}$, $C_{18:2n6t}$, $C_{18:2n6c}$, $C_{20:0}$, $C_{20:1}$, $C_{21:0}$, $C_{20:4n6}$ and $C_{22:0}$) plus the ΣMUFA content. The linear classification model allowed classifying correctly 98% of the original data grouped samples (Fig. 5) and only 71% of the sample for the LOO-CV procedure (individual true positive percentages of 50–100%; and, true negative percentages varying between 55 and 100% for LOO-CV, being the majority of the misclassifications observed between the cheeses

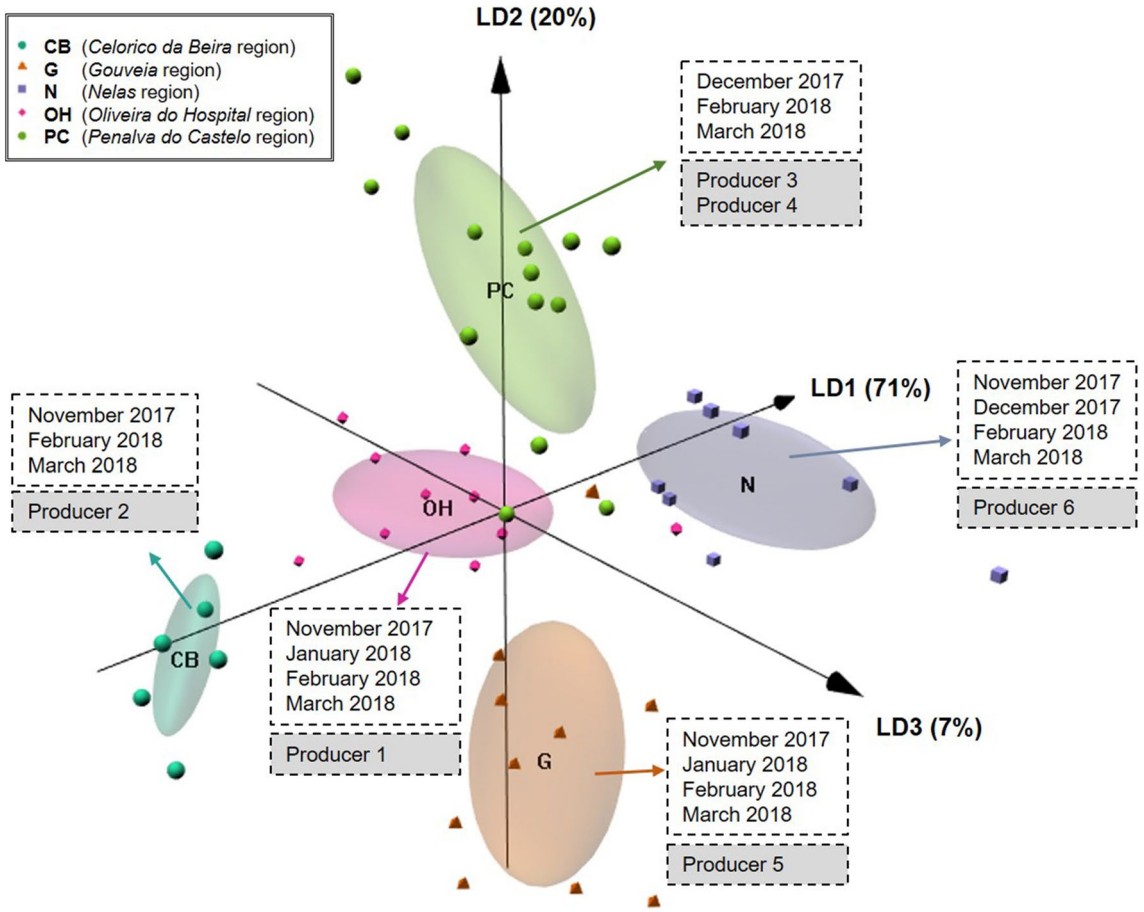

Fig. 4 LDA-SA model classification of Serra da Estrela PDO cheeses (24 cheeses × 2 independent samples) according to the production geographical origin (municipalities of Celorico da Beira—CB, Gouveia—G, Nelas—N, Oliveira do Hospital—OH, and Penalva do Castelo—PC), independently of the cheese producer (six producers) or the production date (five months, ranging from November 2017 to March 2018): 3D plots of the first three most significant discriminant functions based on relative abundance of 12 fatty acids ($C_{6:0}$, $C_{8:0}$, $C_{11:0}$, $C_{12:0}$, $C_{15:0}$, $C_{16:0}$, $C_{16:1}$, $C_{17:0}$, $C_{18:1n9c}$, $C_{18:2n6t}$, $C_{21:0}$ and $C_{20:4n6}$) and the ΣMUFA, selected using the SA algorithm

produced in February and March of 2018). The high number of fatty acids needed to be included in the final LDA-SA model as well as the lower overall sensitivity achieved for the internal-validation procedure pointed out the highest complexity of classifying Serra da Estrela cheeses according to the production dates, which could be tentatively attributed to the fact that the five time-periods evaluated corresponded to five consecutive months (between November 2017 and March 2018), being expected a better classification rate if cheeses were produced in different seasons. Nevertheless, the overall results are satisfactory showing the practical possibility of using the fatty acids profile to discriminate among cheese production dates.

Taking into account the overall reported results, it should be emphasized that, although cheeses production fulfilled the legal PDO regulations, the chemical characteristics of Serra da Estrela cheeses, namely the fatty acids contents, show a high variability, depending on the Producer and production date, which may point out the need of a better production control, in order to standardize the cheese quality, making it easier to detect possible fraudulent practices.

Finally, to demonstrate the feasibility of the proposed approach, the study should be extended to other PDO cheeses, produced from different types of milk. In the near future, the identification of other chemical compounds (e.g., amino acids, volatile compounds among others) as possible cheese origin biomarkers would be a challenging but important task.

Conclusions

This study demonstrated that the fatty acids profiles, besides contributing for the nutritional characteristics of cheese they may be used as chemical biomarkers for assessing the origin and production date of Serra da Estrela PDO cheeses, which is of major importance for producers and consumers. It should be remarked that the fatty acids with the main

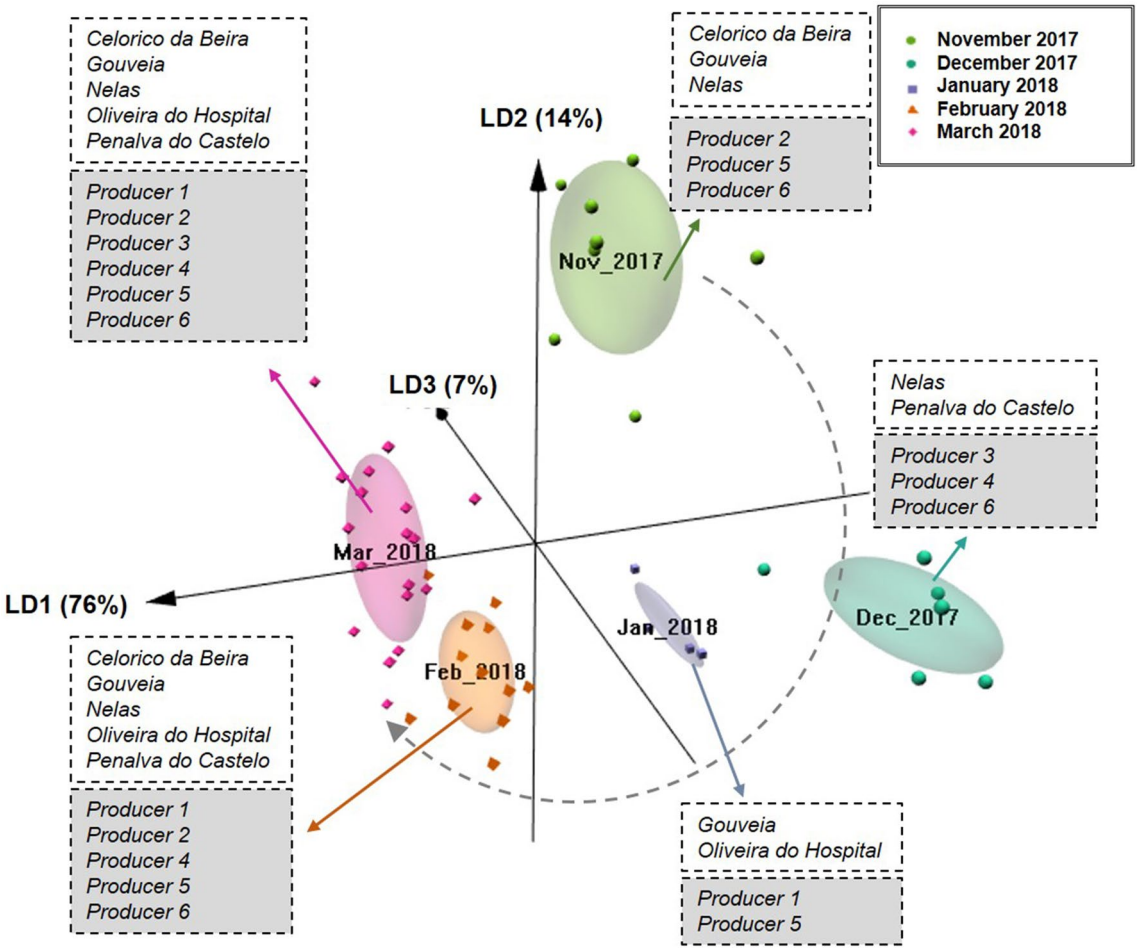

Fig. 5 LDA-SA model classification of Serra da Estrela PDO cheeses (24 cheeses × 2 independent samples) according to the production dates (November 2017 to March 2018), independently of the cheese producer (six producers) or the production geographical origin (five different municipalities within the delimited PDO region): 3D plots of the first three most significant discriminant functions based on relative abundance of 20 fatty acids ($C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{11:0}$, $C_{12:0}$, $C_{13:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:1}$, $C_{17:0}$, $C_{18:0}$, $C_{18:1n9c}$, $C_{18:2n6t}$, $C_{18:2n6c}$, $C_{20:0}$, $C_{20:1}$, $C_{21:0}$, $C_{20:4n6}$ and $C_{22:0}$) and the ΣMUFA, selected using the SA algorithm

discriminant power include saturated, mono- and polyunsaturated fatty acids, being not restricted to the usually most abundant one. In fact, less abundant fatty acids (e.g., $C_{15:0}$, $C_{16:1}$, $C_{17:0}$, $C_{20:1}$, $C_{21:0}$ and $C_{20:4n6}$) seem to play an important role as cheese origin biomarkers. This finding may allow their use for marketing purposes, being foreseen as a practical tool for cheese producers to guarantee brand genuineness and so, to enhance the consumers' confidence when purchasing this type of high-value and appreciated Portuguese cheese. In fact, consumers have always shown preferences for specific cheese brands that are linked to a specific *terroir*.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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